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Cytopathology of *Bombyx mori* (Lepidoptera: Bombycidae) larva integument infected by *Bombyx mori* nucleopolyhedrovirus

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ABSTRACT. *Bombyx mori* nucleopolyhedrovirus (BmNPV) is an entomopathogenic virus of the family Baculoviridae that causes disease in the silkworm, *Bombyx mori*, leading to a series of metabolic disorders that severely affect silk production. The main external sign of BmNPV infection can be observed in the integument of infected caterpillars, which is an important diagnostic indicator that can help direct control measures to prevent viral dispersion in rearing facilities. We analyzed the integument of *B. mori* caterpillars infected with BmNPV in a cytopathological study of epidermal cells and cuticle, to help identify signs of infection. Fifth instar larvae were inoculated with viral suspension and integument segments were processed for light microscopy and transmission electron microscopy. Signs of infection were monitored daily until cocooning. Cytopathology of epidermal cells showed signs of baculovirus infection, leading to cytolysis and release of viral polyhedra into the hemocoel, similar to what occurs in other BmNPV target cells. Considerable modifications were observed in the apical region of the epidermal cells, with disarrangement and involution of the microvilli, and loss of contact of the plasma membrane plaques

with the endocuticle. Plasma membrane plaques are involved in the transport of precursors for chitin synthesis and assembly of chitin myofibrils. The disorganization of the integument of silkworms infected with BmNPV shows the fragility of this organ, which easily ruptures due to infection, leading to the release of BmNPV polyhedra to the external environment, perpetuating the infection.

Key words: Baculovirus; Cytopathology; Cuticle; Ultrastructure; Larval integument cells; *Bombyx mori*

INTRODUCTION

Bombyx mori (Lepidoptera: Bombycidae), known as the mulberry silkworm, is a holometabolous insect totally domesticated by man and used as a biological model in scientific studies (Abdelli et al., 2018). In Brazil, as in other countries, this insect presents great economic importance for silk production. However, the activity suffers with losses in its production, caused mainly by diseases in the breeding sheds, a problem of worldwide proportion (Wang et al., 2016; Bueno et al., 2019).

Among the pathogens that infect *B. mori* are those of viral origin, and in the state of Paraná, Brazil a virus of the Baculoviridae family was identified as one of the main causes of losses in silk production. The *Bombyx mori* nucleopolyhedrovirus (BmNPV) causes grasserie disease (Brancalhão et al., 2002). BmNPV lineage was sequenced by Ardisson-Araújo et al. (2014).

Baculovirus is a large virus family that infect insects, mainly of the order Lepidoptera; it is phylogenetically divided into the genera *Betabaculovirus*, *Deltabaculovirus*, *Gammabaculovirus* and *Alphabaculovirus* (Rohrmann, 2019). BmNPV is a *Alphabaculovirus* (AlfaBV) it consists of a double-stranded circular DNA, associated with capsid proteins, forming the nucleocapsid (Rohrmann, 2019). A lipoprotein envelope surrounds the nucleocapsid, forming the single subgroup (SNPV) when alone, or "multiple" (MNPV), when several nucleocapsids are enveloped, considered the most virulent type (Brancalhão et al., 2009). All this viral content remains inside an occlusion body of polyhedral shape, composed of polyhedrin, which gives protection to the virions against deactivation in an unfavorable environment (Rohrmann, 2019). There are two distinct viral forms of nucleocapsids, the ODVs (occlusion-derived viruses) and the BVs (budded viruses). Although these BV and ODV virions are considered genotypically identical they play different roles in the infectious cycle, differing in morphology and protein composition, origin of viral envelopes, mode of penetration into the host cell and infectivity (Oliveira et al., 2006).

In the larval stage, *B. mori* is particularly susceptible to BmNPV, and infection occurs mainly orally, by ingestion of food contaminated with viral polyhedra (Ribeiro et al., 2009a; Vessaro-Silva et al., 2019). BmNPV is polyorganotrophic, and several tissues have already been recognized as targets (Torquato et al., 2006a; Brancalhão et al., 2009; Ribeiro et al., 2009a; Baggio, et al., 2014; Vessaro-Silva et al., 2019).

The most characteristic external signs of BmNPV infection are the changes that occur in the caterpillar integument, such as alterations in its coloration, resulting from the lysis of infected fat cells that release phospholipids, cholesterol, and fatty acids into the hemolymph, together with mature viral polyhedra. With advancement of infection, the

tegument becomes fragile, mainly due to the hydrolytic enzymes viral cathepsin (V-Cath) and viral chitinase (V-CHIA) (Ishimwe et al., 2015; Bueno et al., 2019), which aid in degradation, culminating in tegument rupture and in the extravasation of the hemolymph with mature polyhedra to the external medium (Ribeiro et al., 2009a). This leads to viral dissemination in the breeding environment of the caterpillars, contributing to the horizontal transmission of the disease (Volkman, 2007).

The integument is formed by the cuticle and underlying epidermal cells that synthesize and secrete the cuticle, which represents the exoskeleton of the insect, being the site of muscle fixation, and is the first line of defense against infectious agents, predators, parasites, and environmental chemicals. In addition, it acts in several metabolic processes, in locomotion, respiration, feeding, excretion, protection against desiccation, behavior, osmoregulation, water control and food reserve (Hopkins and Kramer, 1992).

Thus, considering the multiple physiological roles played by the *B. mori* integument, including its action on viral dissemination, it would be useful to analyze its cytopathological response to BmNPV infection. Moreover, considering that in the tegument structure pathological alterations become perceptible, the organ can be used as an important indicator for the detection of the disease in the field. This makes it possible to separate and dispose of infected insects, potential spreaders of the viral disease in breeding sheds.

MATERIAL AND METHODS

Insects

The experiments were carried out with a commercial hybrid of *B. mori* (Lepidoptera: Bombycidae) obtained from the silkworm industry BRATAC S.A., Paraná, Brazil, and kept in nursery rooms, receiving fresh mulberry (*Morus* sp.) leaves daily (Ribeiro et al., 2009b).

Viral inoculation

The BmNPV inoculum, developed by (Brancalhão et al., 2002), was quantified in Neubauer chambers at a concentration of 1.70 x 10^7 POBs/mL (POBs: Polyhedral Occlusion Bodies). For inoculation, the caterpillars were kept individually confined, after moulting to the 5th instar. They were fed with leaf discs (diameter 2 cm^3), previously sprayed with 10 μL of viral suspension. The controls were fed with leaf discs sprayed with filtered water. After feeding the caterpillars were transferred to breeding rooms receiving leaves free of BmNPV, three times a day until the beginning of cocoon formation.

Confirmation of BmNPV infection was determined by monitoring the signs presented by the caterpillars (Ribeiro et al., 2009b), changes in tegument color and swelling of segmental membranes, and presence of viral polyhedra in the hemolymph (Senem et al., 2016).

Light Microscopy and Transmission Electron Microcopy

From 1 to 7 days post-inoculation (dpi), at 24-hour intervals, with the aid of a stereoscope microscope, three caterpillars from the control group and three from the

inoculum were anesthetized by exposed at -20°C and immediately the caterpillars were transferred to glass container with a lid containing a cotton ball embedded with ethyl ether during 5 min. After that the caterpillars were dissected and segments of the integument were removed and fixed.

For light microscopy, the integument was fixed for 24 h at 4°C and followed routine histological processing for paraffin embedding (Brancalhão et al., 2009). The 5 μm slices were obtained on an Olympus CUT4055 microtome and stained by the modified Azan technique for viral occlusion bodies (Hamm, 1966) for identification of cytopathologic events. The material was analyzed under an Olympus BX60 light microscope and the regions of interest were photomicrographed.

For transmission electron microscopy the integument was fixed in 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.2). The samples remained in fixative solution for 24 h and were post-fixed with 1% osmium tetroxide in the same buffer for 2 h; they were then contrasted in 0.5% uranyl acetate for 1 h at room temperature. Dehydration occurred by increasing the acetone concentration (50, 70, 80, 90%, and 100%) up to inclusion in Polybed 812 resin. Ultrathin slices obtained were tested in uranyl acetate and lead citrate and analyzed with a JEOL JM 1400 electronic transmission microscope, at the Research Support Center Complex (COMCAP) of the State University of Maringá, UEM.

RESULTS AND DISCUSSION

The first external signs of BmNPV infection in the *B. mori* caterpillars occurred from the fifth dpi, with a change in tegument coloration from white to yellow-white. These signs were followed by a rapid evolution of the symptoms, with swelling of the intersegmental membranes and milky liquid trace, caused by ruptures in the integument, and death of more than 50% of the caterpillars within 48 h, after the appearance of the first signs. In addition, there was prolongation of the larval stage, with duration of 11 days for the inoculated caterpillars, while the control group had larval stage duration of nine days.

Cytopathological analysis of BmNPV-infected *B. mori* cells also revealed the onset of infection the fifth dpi, with hypertrophic epidermal cell nuclei, presence of viroplasm and viral polyhedra at various stages of the infectious cycle, indicating asynchronous appearance (Figure 1A). During the infection enveloped nucleocapsids are produced, the polyhedrin polymerizes around them, initiating the formation of the polyhedron that is still without a defined format (Figure 2A), which calls it immature polyhedron. When mature, polyhedra acquire a membranous envelope originating from the inner nuclear membrane (Figure 2A), attaining the characteristic geometric shape (Figure 1B and 2B) of truncated octahedral. Under light microscopy, the polyhedrons were stained red (Figure 1B and 1C). Figures 1D and 2C show the integument of the control group for comparison. Electron-lucent regions are visualized in both control and infected groups throughout the cytoplasm of *B. mori* epidermal cells (Figure 2B and 2C).

The cuticle and the epidermis of silkworm undergo a remarkable change during BmNPV infection and ultrastructural cytopathological aspects of the integument revealed deep alterations in the apical region of cubic epithelial cells (Figure 1B and 2B), with the disorganization of microvilli, leading to derangements and involutions (Figure 3A). In addiction, the contact of the plasma membrane plaques with the endocuticule, perfectly

arranged in the normal state (Figure 2C and 3B), where in many regions of the infected integument they were no longer visible (Figure 3A). The lamellae arranged of chitin fibrils in the endocuticular region is lost (Figure 1C and 3A), resulted in openings between the epithelium and the endocuticle (Figures 1B and 2B), resulting in a fragility in the integument and the lysis of the epidermal cells (Figure 1C), in the insect infected by BmNPV.

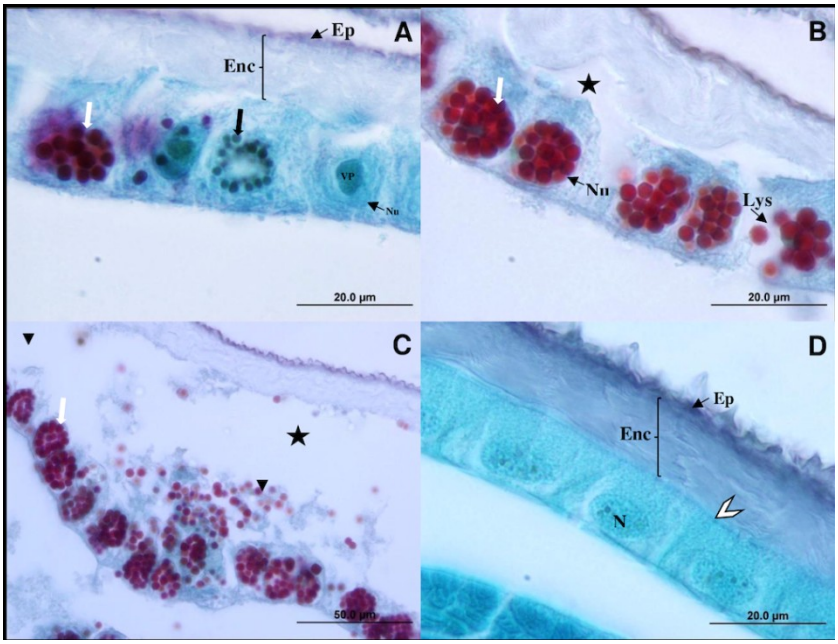

Figure 1. Microscopical optical images of the *Bombyx mori* 5th instar larva integument infected with BmNPV in longitudinal sections stained with modified Azan. (**A)** epidermal cells with hypertrophic nucleus (Nu); viroplasma (VP); immature polyhedra in formation (black arrow) and mature in red color (white arrow). In the apical cuticle region: the endocuticle (Enc) and epicuticle (Ep). (**B)** viral polyhedra and initiations of cell lysis (Lys), disarrangement of the endocuticle and its detachment from the epidermal cells (Star). (**C)** disintegration of the integument with release of the polyhedra (▼). (**D)** control material, local cytoplasmic plaques (white arrowhead).

Integument alterations are the most recurrent signs of BmNPV infection, as observed by Ribeiro et al. (2009b). However, in the present study, the symptomatic evolution was faster in comparison to that described by the authors. Different intervals of evolution of the disease are common in the infections caused by BmNPV, these variations may be related to: the inoculum, due to the differentiated virulence of viral isolates and amount of polyhedra ingested by the host (Zhang et al., 2012); the climate change of temperature, humidity and pollution (Li et al., 2015); the establishment of the caterpillars, by the diet, the quality and availability of the mulberry leaves offered and the number of individuals per box of breeding (UI-Haq et al., 2014; Thulasi and Sivaprasad, 2015); and the strains and hybrids used, which present differences in resistance and susceptibility to pathogens (Pereira et al., 2013; Zhou et al., 2013).

Another disease marking was the increase of larval cycle duration, also observed by Brancalhão et al. (2009) and Ribeiro et al. (2009b) in *B. mori* caterpillars infected with

BmNPV. This prolongation of the larval stage is related to the activity of the ecdysteroid viral enzyme UDP-glycosyltransferase (EGT gene), which, when expressed, catalyses the transfer of a monosaccharide (UDP-glucose or UDP-galactose) to the ecdysone hormone, causing its inactivation. Thus, the caterpillars do not make molt, continue to feed, resulting in increase in viral progeny (Bianchi et al., 2000). This EGT gene is present in many baculoviruses, which explains the prolongation of the larval stage in the BmNPV-infected group compared to the control group (Simón et al., 2012).

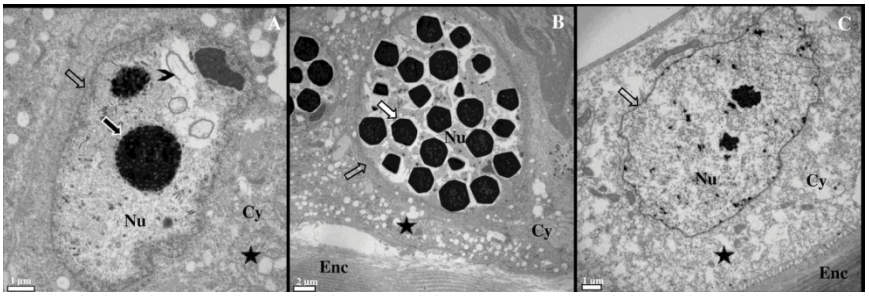

Figure 2. Transmission electron micrograph images of the *Bombyx mori* 5th instar larva integument infected by BmNPV. Integument epidermal cells, the nuclear envelope (hollowed arrow), nucleus (Nu), cytoplasm (Cy) and electron-lucent regions (Star). (**A)** hypertrophic nucleus with BmNPV polyhedra in formation (black arrow), polyhedron envelope (black arrow tip), (**B)** mature polyhedra (white arrow), endocuticle (Enc). (**C)** control material.

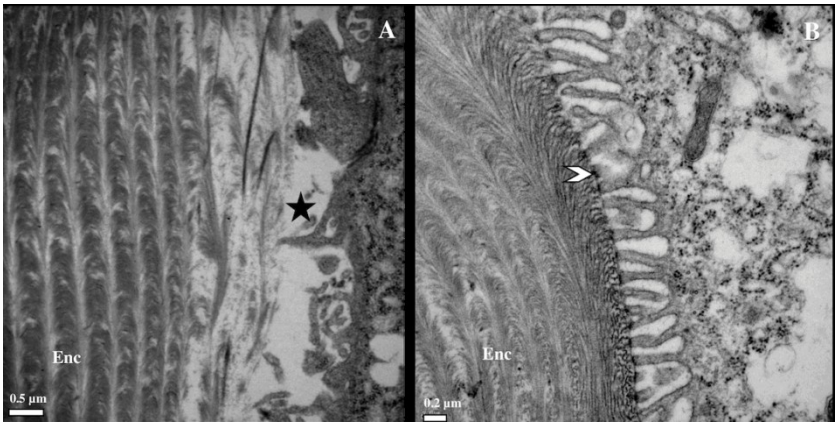

Figure 3. Transmission electron micrograph images of the *Bombyx mori* 5th instar larva integument infected by BmNPV. (**A)** infected 7th day post-inoculation cells, disruption of microvilli and plasma membrane plaques (Star), culminating with detachment of epidermal cells from endocuticle (Enc). (**B)** control material, epidermal cells with microvilli and plasma membrane plaques (white arrowhead).

During the infection, the first visible viral structure was the viroplasm or virogenic stroma, where nucleocapsid production occurs, initially without the envelope and subsequently enveloping it to form enveloped nucleocapsids or virion (Brancalhão et al., 2009; Senem et al., 2016). Rohrmann (2019) reports that the nucleocapsid envelope originates from segments of the internal nuclear membrane associated with viral proteins. Variations in the shape and size of polyhedra can also be visualized, being common to AlfaBV and dependent on the amount of nucleocapsids included, metabolism of the

infected cell, genetic variability and even visualization plane under microscopy (Torquato et al., 2006b; Cheng et al., 2013a; Chaeychomsri et al., 2015).

In addition to this diversity, baculovirus mutant polyhedra can form cubic occlusion bodies after several passages of the viral inoculum either in cultured cells or in the insect organism (Cheng et al., 2013b). The serialized passageway may also lead to the accumulation of mutant polyhedra in the inoculum, and a decrease in the amount of nucleocapsids contained within the polyhedron, reducing virulence (Chakrabarty et al., 2012). In our study, the virulence of the inoculum was considered high, due to the mortality rate and the rapid death of the caterpillars after manifestation of the first signs of disease, only two days after the beginning of the signs and more than 50% died due to infection by the BmNPV.

Cytopathological analysis showed cuticle disorganization during the BmNPV infection in the tegument cells, with the disarrangement of its layers, endocuticle and epicuticle, which separated from the underlying epithelium. The structure of the cuticle is controlled and precisely regulated by the epidermal cells and this disorganization, resulting from the alteration of the larval metabolism by the viral infection, also destabilizes the contact of the plasma membrane plaques with the endocuticule (Lamer and Dorn, 2001). Plasma membrane plaques are involved in the transport of precursors for chitin synthesis and assembly of chitin myofibrils, fundamental to bind cuticle and epidermis together, and where new cuticular material is assembled and added to the already existing cuticle (Doucet and Retnakaran, 2012).

The openings in the endocuticular region may have occurred due to breaks in the molecular bonds of chitins and microfibrils, in the plasma membrane plaques of microvilli. Smagghe et al. (1996) observed alterations in the microvilli and lamellar pattern of *Spodoptera exigua* exposed to tebufenozoide, an insecticide that acts as an agonist of ecdysteroids, steroids that influence the process of ecdysis. Similarly, Oh et al. (2013) observed irregularities in the lamellar cuticle and presence of chitinases between these lamellae in *Pieris rapae* (Lepidoptera: Pieridae), infected with granulovirus.

Openings in the integument are characteristic of insect moults, where the process of apolysis separates the endocuticle and the epithelial layer forming the apolysial space (Merzendorfer and Zimoch, 2003). Oh et al. (2013) visualized the partial rupture of the basement membrane in granulovirus infected *Pieris rapae* epidermal cells, the same occurring in this study (not shown). Mansour et al. (2012) reported on the lepidopteran *Plutella xylostella*, treated with allyl isothiocyanate, organosulfur compound and natural repellent, epidermal cells indistinguishable and almost completely destroyed, separated from the endocuticle by openings in the apolisial space.

Transmission electron micrograph images of the epidermal cells revealed electron-lucent regions in both groups. Hu et al. (2013) relates these electron-lucent and ellipsoidal regions to urate granules present in cells of the epidermis of mutant lines of caterpillars with white coloration, which is the case of the hybrid used. These granules from the greasy body are accumulated in the tegument to structure the new cuticle during metamorphosis, and are then transported to hemolymph and excreted by the caterpillar with the beginning of its movement to the woods, causing the gradual change from white to translucent (Kato et al., 2006).

The cuticle disruption caused by BmNPV infection contributes to the fragility and destabilization of the caterpillar integument, observed from the seventh dpi, when lysis of

the epidermal cells was also visualized. This integumentary weakness caused by viral infection makes it impossible to protect and maintain the anatomical integrity of the cuticle, and can cause damage to the functions of joint attachments that allow movements in responses to changes in the environment; it also impacts on organs and glands responsible for the production of hormones (Andersen, 2010). Due to the tissue characteristics of the organ, the microvilli plaques and the lamellae of the endocuticle are disrupted, causing rupture of the integument, which leads to the release of the viral polyhedra into the external environment, contributing to viral dissemination, resulting in disease transmission.

Analysis of the integument of the silkworm larvae infected by BmNPV showed epidermal cell cytolysis with disarrangement and involution of the microvilli, loss of contact of the plasma membrane plaques with the endocuticle, leading to release of viral polyhedra from the larva hemolymph to the external environment.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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